Influence of *Bacillus subtilis* phoR on cell wall anionic polymers

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In *Bacillus subtilis* the Pho regulon is controlled by a sensor and regulator protein pair, PhoR and PhoP, that respond to phosphate concentrations. To facilitate studies of the Pho regulon, a strain with an altered PhoR protein was isolated by *in vitro* mutagenesis. The mutation in this strain (phoR12) leads to the production of a PhoR sensor kinase that, unlike the wild-type, is functionally active in phosphate-replete conditions. The lesion in phoR12 was shown to be a single base change that results in an Arg to Ser substitution in a region of PhoR that is highly conserved in histidine sensor kinases. While a phoR-negative mutant was unable to induce the synthesis of cell wall teichuronic acid under phosphate-limited conditions, the *phoR12* mutant showed a relative increase in teichuronic acid and a decrease in teichoic acid, even under phosphate-replete conditions. The latter suggests that some or all of the genes required for teichuronic acid synthesis are members of the Pho regulon.

Keywords: phosphate stress, histidine sensor kinase, response regulator, alkaline phosphatase, signal transduction

INTRODUCTION

Phosphorus is a major growth-limiting substrate in natural environments such as soil (Hulett, 1993). When the growth of *Bacillus subtilis* becomes limited by the availability of phosphorus, a programmed series of responses is induced that can ultimately lead to sporulation. Among the earliest of these responses are processes that reduce the cell’s requirement for phosphorus, increase its affinity for the uptake of phosphate and lead to the production of extracellular hydrolytic enzymes that allow the cell to recover inorganic phosphate from organic sources such as teichoic acids (Grant, 1979; Eymann et al., 1996). If these conservation and scavenging mechanisms are effective, the developmental pathway leading to sporulation is reversed and the organism resumes vegetative growth.

*B. subtilis* responds to phosphate stress by the coordinated induction of approximately 20 genes (Eymann et al., 1996). Prominent among these are genes of the Pho regulon (Seki et al., 1987; Hulett, 1993; Hulett et al., 1994a, b), whose products include alkaline phosphatases (APase), alkaline phosphodiesterase (APDase), a high-affinity phosphate transporter and, as reported in this paper, proteins involved in teichuronic acid synthesis. The phosphate-binding lipoprotein component, YzmB, of a high-affinity phosphate transporter (YzmCDEF) is the major protein induced under these conditions (Eymann et al., 1996). The genes encoding APDase (Le Hegarat & Anagnostopoulos, 1973; Yamane & Maruo, 1978) have not been identified; however, B. *subtilis* has an APase multigene family (Eymann et al., 1996). Two APase genes responsible for 95% of APase activity have been mapped and sequenced (Hulett et al., 1991). The main APase, the product of the *phoA* gene (previously *phoAIV*), is induced specifically in response to phosphate starvation (Kapp et al., 1990). A second APase, the product of the *phoB* gene (previously *phoAll*), is induced in response to phosphate stress and during sporulation (Chesnut et al., 1991; Birkey et al., 1994).

The Pho regulon of *B. subtilis* is controlled by two trans-acting regulators that together form the components of...
a two-component, environment-sensing, signal-transduction system (Seki et al., 1987, 1988). The components of this system, PhoP and PhoR, are equivalent, respectively, to the PhoB and PhoR proteins of Escherichia coli (Tommassen et al., 1982; Makino et al., 1985, 1986). In E. coli, PhoR is a membrane-spanning sensor protein with histidine protein kinase (HPKase) and phosphatase (PTase) activities (Makino et al., 1989; Scholten & Tommassen, 1993). Activation at low phosphate concentrations leads to the production of PhoR ~ P which, in turn, acts as a substrate for the activation of the response regulator PhoB, required for the induction of the Pho regulon (Stock et al., 1989; Parkinson & Kofoid, 1992). Similarities between the E. coli and B. subtilis components and the genomic organization of their genes suggest that their Pho regulons function in a similar manner (Seki et al., 1987, 1988). If cultures continue to experience growth-limiting phosphate stress, a second response regulator, Spo0A ~ P, terminates the phosphate response and initiates sporulation (Jensen et al., 1993). Recently, a third signal-transduction system, ResD/ResE, has been shown to be required for full induction of the Pho regulon (Hulett, 1995).

An important mechanism for conserving phosphate in B. subtilis involves changes to the composition of the cell wall. Under phosphate-replete conditions the cell wall contains as much as 15% of total cell phosphorus (Archibald et al., 1993). The wall phosphorus is a component of teichoic acids, which constitute nearly 50% of the wall by weight. When B. subtilis is grown under phosphate-limiting conditions, a significant proportion of the wall teichoic acid is replaced by teichuronic acid (Ellwood et al., 1969; Lang et al., 1982), a phosphate-free anionic polymer. Not only does this reduce the cell's requirement for phosphorus, but teichoic-acid-containing cell wall released into the growth medium (Merad et al., 1989) represents a significant potential source of phosphorus (Grant, 1979) that could be recovered by the combined activities of APase and APDase. Whilst there is little information on the regulatory mechanisms that lead to the replacement of one type of anionic polymer by another, the rate of teichuronic acid synthesis correlates well with that of APase synthesis (Mauck & Glaser, 1972). Taken together with data in this paper, this suggests that genes responsible for teichuronic acid biosynthesis are also components of the Pho regulon.

**METHODS**

**Strains and plasmids.** These are listed in Table 1.

**Media and reagents.** TY medium contained 1% Bacto tryptone, 0.5% yeast extract and 1% NaCl solidified, if necessary, with 1.5% Difco agar. Minimal medium, required for the transformation of B. subtilis, consisted of Spizizen's minimal salts (Spizizen, 1958), supplemented with glucose (0.5%), casein hydrolysate (0.02%) and required amino acids (20 µg ml⁻¹). Chemostat medium (CHMM) comprised: 30 mM K₂SO₄, 50 mM (NH₄)₂SO₄, 1 mM citric acid, 5 mM glutamic acid, 0.1 mM FeCl₃, 0.1 mM CuCl₂, 37.5 µM MgCl₂, 25 µM ZnCl₂, 25 µM MnCl₂, 5 µM CuCl₂, 5 µM CoCl₂, 5 µM Na₂MoO₄, with 30 mM glycerol as carbon source and 1 mM tryptophan, methionine and phenylalanine. Phosphate, added as NaH₂PO₄, was 5 mM in the magnesium-limited medium and 0.25 mM in the phosphate-limited medium. To analyse the induction of APase and APDase, batch cultures were grown in low- (LPDM) or high-phosphate (HPDM) defined medium, as modified from Hulett et al. (1990): 50 mM Tris (pH 7-1), 3.03 mM (NH₄)₂SO₄, 6.8 mM trisodium citrate, 3.04 mM FeCl₃, 1 mM MnCl₂, 3.5 mM MgSO₄, 0.01 mM ZnCl₂, 0.5% glucose, 0.05% Casamino acids, 10 mM L-arginine, 0.2 mM KH₂PO₄ to 3.5 mM (HPDM) or 0.25 mM (LPDM). When required, ampicillin (80 µg ml⁻¹), kanamycin (20 µg ml⁻¹) or chloramphenicol (5 µg ml⁻¹) was added to the media. IPTG was obtained from Serva. p-Nitrophenol phosphate (pNPP), bis-p-nitrophenol phosphate (bis-pNPP) and hydroxyamine were from Sigma.

**Chemostat cultures.** Chemostat cultures were inoculated with batch cultures (OD₆₀₀ 0.5) of CHMM chemostat medium (P, 5 mM) in a 1 l fermentation vessel (LH Engineering). The air flow was set at 21 min⁻¹ and the impeller speed at 500 r.p.m. The temperature was maintained at 37 °C and the pH at 7.0 using 2 M NH₄OH and 1 M HCl. Addition of medium was controlled by a peristaltic pump (Watson-Marlow) at a flow rate of approximately 200 ml h⁻¹ (equivalent to a mean generation time of approximately 3.5 h). Growth was monitored by determining the dry weight of the cells retained on pre-weighed cellulose acetate membrane filters (pore size 0.45 µm; Whatman).

**DNA techniques.** Procedures for DNA purification, restriction endonuclease digestion, ligation, transformation of E. coli TG1, agarose gel electrophoresis and Southern transfer of DNA to Hybond N (Amersham) were carried out as described by Sambrook et al. (1989). Competence of B. subtilis was induced as described by Bron & Venema (1972). For DNA amplification, a Taq polymerase with proof-reading activity (Perkin-Elmer) was used. Restriction endonucleases and other enzymes were from NBL Gene Sciences.

Oligonucleotides were synthesized using a Beckman Oligo 1000. The following oligonucleotides were used (the numbers refer to those in Seki et al., 1988): (i) upstream primer, PRX1 5' CGGTTCAGATTTA AGTTCGACC 3', localized 5' to the ribosome-binding site of phoR, and incorporating a XbaI restriction site; (ii) downstream primer, PRP2 5' CTACCTGCAGAGGT TAAACGTGCAG 3', localized 3' to the phoR stop codon, and incorporating a PsI restriction site.

Mutagenesis of plasmid DNA with hydroxyamine was carried out in sodium phosphate buffer (pH 6.0, 1 mM EDTA) containing 0.2 M hydroxyamine. Circular plasmid DNA was incubated for 45 min on ice and then heated for 60 min at 65 °C. After the reaction period, the DNA solution was diluted fourfold with water and dialysed against TE-buffer (Sambrook et al., 1989). The mutagenized plasmid DNA was amplified initially in E. coli TG1, extracted and then transformed into B. subtilis 168 using natural competence.

**Enzyme assays.** APase production by colonies growing on agar plates was determined in situ (Yamane & Maruo, 1978). After overnight incubation at 37 °C, agar plates were overlaid with sterile filter paper (Whatman) wetted with pNPP solution (1 mg ml⁻¹ pNPP in 1 M Tris pH 8.0). Colonies showing APase activity were identified by the development of a bright yellow colour resulting from the hydrolysis of pNPP. APase activities of cultures growing in liquid media were determined as described by Nicholson & Setlow (1990). One unit of enzyme activity was defined as the amount of enzyme
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Bacterium or plasmid</th>
<th>Genotype/characteristics</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG1</td>
<td><em>hsdD5/F traD36 proA</em>B* Δ(lac-pro) lacI* lacZΔM15</td>
<td>Carter &amp; Winter (1985)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td><em>trpC2</em></td>
<td></td>
</tr>
<tr>
<td>1A96</td>
<td><em>pheA1 trpC2</em></td>
<td></td>
</tr>
<tr>
<td>1A306</td>
<td><em>metC3 phoR2 tal-1</em></td>
<td></td>
</tr>
<tr>
<td>GCH635</td>
<td><em>gtaB</em> (phage 25-resistant mutant of 168, linked to hisA3 by transduction and partially reversed by the addition of galactose)</td>
<td></td>
</tr>
<tr>
<td>GCH871</td>
<td>168::pDYphoR12 (P&lt;sub&gt;phoR&lt;/sub&gt; P&lt;sub&gt;spac&lt;/sub&gt;phoR, Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>GCH872</td>
<td>168::pDKphoR12 (P&lt;sub&gt;phoR&lt;/sub&gt; P&lt;sub&gt;spac&lt;/sub&gt;phoR, K&lt;sup&gt;m&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>GCH873</td>
<td>168::pDYphoR (P&lt;sub&gt;phoR&lt;/sub&gt; P&lt;sub&gt;spac&lt;/sub&gt;phoR, Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>GCH875</td>
<td>168::pDKphoR (P&lt;sub&gt;phoR&lt;/sub&gt; P&lt;sub&gt;spac&lt;/sub&gt;phoR, K&lt;sup&gt;m&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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</tr>
<tr>
<td>pUC119</td>
<td><em>E. coli</em> cloning vector</td>
<td>Vieira &amp; Messing (1987)</td>
</tr>
<tr>
<td>pDY6</td>
<td><em>E. coli</em> plasmid containing P&lt;sub&gt;spac&lt;/sub&gt; promoter and lacI repressor gene, Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>R. Breitling, University of Jena</td>
</tr>
<tr>
<td>pDYphoR</td>
<td>pDY6 derivative with phoR under P&lt;sub&gt;spac&lt;/sub&gt; control, Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pDKphoR</td>
<td>pDY6 plasmid with Cm&lt;sup&gt;r&lt;/sup&gt; replaced with Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pDYphoR12</td>
<td>pDY6 derivative with phoR12 under P&lt;sub&gt;spac&lt;/sub&gt; control, Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pDIA5304</td>
<td><em>E. coli</em> cloning vector, Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>P. Glaser, Institut Pasteur</td>
</tr>
<tr>
<td>pDIAphoR12</td>
<td>pDIA5304 derivative with the 250 bp 3' terminal end of phoR12, Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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</table>

**RESULTS**

**Cloning and inducible expression of phoR**

The *phoR* gene was amplified by PCR from chromosomal DNA of *B. subtilis* 168 using primers PRX1 and PRP2. The product was digested with XbaI and PstI and cloned into pUC119. The fidelity of the insert was confirmed by DNA sequencing and then the XbaI–PstI *phoR* fragment was transferred to pDY6, generating pDYphoR (Fig. 1), where it was shown to be under the control of the IPTG-inducible P<sub>spac</sub> promoter (data not shown).

Plasmid pDYphoR was used to integrate a second copy of the *phoR* gene into the chromosome of *B. subtilis* 168 via a Campbell-like recombination event within *phoR* (Fig. 1), generating strain GCH873 (168::pDYphoR). The appropriate mode of integration, in which one of the *phoR* genes was under the control of the P<sub>spac</sub> promoter while the other was under the control of its natural promoter P<sub>phoPR</sub>, was confirmed by Southern hybridization.

The pattern of *phoR* expression in GCH873 was verified by monitoring the induction of APase activity during phosphate starvation (Fig. 2). The inducibility of APase in GCH873 was similar to that of its parent, strain 168, except that the level of expression was somewhat higher, possibly reflecting the increase in copy number. No APase activity was detected under phosphate-replete conditions, irrespective of the presence of IPTG, confirming that induced wild-type PhoR is not functionally active in *B. subtilis* under phosphate-replete conditions.

**Isolation of a mutant of *B. subtilis* showing constitutive APase activity**

In order to isolate mutants in which PhoR shows functionally constitutive activity, i.e. in which the phosphorus starvation signal is active in phosphate-rich medium, plasmid pDYphoR was subjected to *in vitro* mutagenesis with hydroxylamine. Mutagenized plasmid DNA was transformed into *E. coli* TG1 and, after
amplification of the equivalent of $10^4$ transformants, plasmid DNA was isolated and integrated into the chromosome of *B. subtilis* 168. Transformants were selected on chloramphenicol-containing TY plates in either the presence or absence of IPTG. Mutants with constitutive APase activity were selected by using the APase plate assay.

A number of presumptive APase constitutive mutants were isolated and one mutant, GCH871 (168::pDYphoR12), was selected for further analysis. Under phosphate-replete conditions (TY medium), GCH871 exhibited APase activity throughout growth (Fig. 3). The addition of IPTG, leading to the induction of the wild-type copy of the *phoR* gene, almost completely reversed the effects of the *phoR12* mutation. GCH873, containing two wild-type copies of *phoR*, showed no APase activity under these conditions, irrespective of the presence of IPTG (Fig. 3).

GCH871 and GCH873 were also grown in HPDM and LPDM and their APase and APDase activities determined. Little or no APase activity was detected with strain GCH873, containing only wild-type versions of the *phoR* gene, at any stage in the growth cycle on HPDM (Fig. 4a). In contrast, GCH871 showed measurable amounts of APase activity throughout growth on HPDM, but the levels were markedly higher in the strain containing *phoR12*. Growth of GCH871 into phosphate starvation in LPDM medium resulted in hyperinduction of both APase and APDase activities with respect to GCH873 (Fig. 4b).

Chromosomal DNA isolated from strain GCH871 was used to transform strain 168 for chloramphenicol resistance. All of the transformants (> 50) exhibited the APase-positive phenotype on phosphate-replete agar,
Mutant phosphate sensor in *B. subtilis*

![Growth, APase and APDase activities of *B. subtilis* strains with the wild-type and mutant (phoR12) copies of the phoR gene, grown in (a) HPDM and (b) LPDM. Growth (○), APase (▲) and APDase (●) activities of strain GCH873 (168::pDYphoR) growth (○), APase (▲) and APDase (●) activities of strain GCH871 (168::pDYphoR12).](image)

Fig. 4.

![Alignment of PhoR proteins from *B. subtilis*, *E. coli*, *Klebsiella pneumoniae*, *Mycobacterium leprae*, *Shigella dysenteriae* and *Synechococcus* sp. (Alba et al., 1993; GenBank U38917) in regions 4 and 5 (Albright et al., 1989; Lee et al., 1989), showing the conserved Arg-Ser-Arg (RSR) residues (bold).](image)

Fig. 5.

confirming that the lesion was linked to the chloramphenicol resistance gene (see Fig. 1).

**Characterization of the mutant phoR gene**

To localize the lesion mediating the altered phenotype, both copies of *phoR* in GCH871 were amplified separately. The downstream copy (Fig. 1) was released from chromosomal DNA by digestion with *Sph*I and subsequently re-circularized by ligation. The released plasmid was transformed into *E. coli*. Similar attempts to release the upstream, *p* _phoPR_-controlled copy of *phoR* by digesting chromosomal DNA with *Xba*I failed to generate transformants in *E. coli*, suggesting that the mutated copy of *phoR* is detrimental for this organism. Consequently, the upstream copy of *phoR* was amplified by PCR with primers PRX1 and PRP2, and the 5′ (*XbaI–EcoRI*) and 3′ (*EcoRI–PstI*) fragments of the product sub-cloned separately into *E. coli* vector pDIA5304.

Both copies of *phoR* from GCH871 were sequenced. A point mutation (A to T transition) was located at position 1679 (Seki et al., 1988) in the upstream (*P* _phoPR_–controlled copy, resulting in a change in amino acid residue 532 from Arg to Ser (Fig. 5). The mutated gene was designated *phoR12*.

The pDIA5304-based derivative containing the *EcoRI–PstI* fragment of *phoR12* (pDIA5phoR12) was integrated into the chromosome of *B. subtilis* 168 via a Campbell-type integration event. The resulting transformants, 168::pDIAphoR12, could be divided into two types: those, like GCH871 (168::pDYphoR12), showing constitutive APase activity (about 50% of the transformants) and the remainder showing the wild-type phenotype. The presence of integrants showing the constitutive APase phenotype confirmed that the altered functionality of *phoR12* was due to a lesion located within the *EcoRI–PstI* 3′-fragment.

**Influence of phoR on the synthesis of anionic polymers**

Wild-type and *phoR12* strains were grown in LPDM and HPDM, their cell walls isolated and anionic polymer content analysed. The walls of *B. subtilis* strain 168 grown in HPDM showed, as expected, high concentrations of teichoic acids and low concentrations of teichuronic acid. In contrast, strain GCH871 showed a three- to fourfold increase in the concentration of teichuronic acid, accompanied by a reduction in teichoic acids (Table 2). Under phosphate starvation conditions, which normally lead to the induction of teichuronic acid synthesis, the *phoR12* mutant showed hyperinduction of teichuronic acid synthesis and a further reduction of teichoic acid synthesis compared with that of the wild-type (Table 2). The total anionic polymer content of cell walls isolated from the *phoR12* mutant was lower than that in the wild-type, both under low- and high-phosphate conditions (Table 2).

Attempts to determine the wall composition of strain GCH871 during steady state in a chemostat were abandoned because the *phoR12* mutation was not stable under these conditions. We therefore constructed a merodiploid in which a wild-type copy of *phoR* on the synthesis of anionic polymers.
Table 2. Analysis of the anionic polymer content of cell walls isolated from the wild-type (168) and the phoR72 (GCH871) mutant of B. subtilis grown to stationary phase in high-phosphate (HPDM) or low-phosphate (LPDM) defined medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Content [µmol (mg cell wall)-1]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Teichoic acid</td>
</tr>
<tr>
<td>168</td>
<td>HPDM</td>
<td>0.84</td>
</tr>
<tr>
<td>GCH871</td>
<td>HPDM</td>
<td>0.49</td>
</tr>
<tr>
<td>168</td>
<td>LPDM</td>
<td>0.56</td>
</tr>
<tr>
<td>GCH871</td>
<td>LPDM</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Table 3. APase activities and cell wall anionic polymer content of GCH872, containing an IPTG-inducible phoR12, after growth to steady state in a chemostat

<table>
<thead>
<tr>
<th>Limiting substrate</th>
<th>Presence of IPTG</th>
<th>APase [units (mg dry wt)-1]</th>
<th>Content [µmol (mg cell wall)-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Teichoic acid</td>
</tr>
<tr>
<td>Mg²⁺-limited</td>
<td>-</td>
<td>0.82</td>
<td>1.71</td>
</tr>
<tr>
<td>Mg²⁺-limited</td>
<td>+</td>
<td>87.93</td>
<td>0.59</td>
</tr>
<tr>
<td>P₁-limited</td>
<td>+</td>
<td>217</td>
<td>0.34</td>
</tr>
</tbody>
</table>

absence of IPTG, only the wild-type copy of the gene is expressed. GCH872 was established under magnesium limitation (P₁-replete) and then IPTG was added to the inflowing medium; the culture was then switched to phosphate limitation, again in the presence of IPTG. Samples were analysed for APase activity and for cell wall composition (Table 3). The data confirmed those observed for GCH871 grown in batch culture. Addition of IPTG to magnesium-limited cultures led to the induction of APase activity, a fourfold increase in teichuronic acid in the cell wall and a three-fold reduction in teichoic acid. A similar pattern was observed after changing to phosphate limitation in the presence of IPTG; the altered cell wall composition was enhanced, although there was a reduction in APase activity. Phosphate starvation is known to trigger sporulation and the lower induced APase activity could be due to the previously observed influence of SpoOA on the expression of phoA (Hulett et al., 1994a; Jensen et al., 1993).

The altered composition of the cell wall in mutants expressing phoR12 led us to investigate the influence of PhoR on cell wall composition at different phosphate concentrations. B. subtilis strains 1A96 (phoR) and 1A306 (phoR2) were grown in chemostats under either phosphate-replete (magnesium-limiting) or phosphate-limiting conditions. In the case of the PhoR wild-type strain, the biomass reached a steady state under phosphate limitation of approximately 0.7 µg (ml culture)-1, while the biomass of the phoR mutant at the same medium phosphate concentration was approximately 0.3 µg ml-1 (Fig. 6). These differences in cell yield presumably reflect the operation, in the wild-type strain, of mechanisms for conserving phosphate and recovering P₁ from organic sources in the growth medium.

In the wild-type, transition to phosphate limitation was accompanied by a transient induction of APase activity and the replacement of teichoic acid in the cell wall with...
the non-phosphate-containing teichuronic acid (Fig. 7a). When the medium was changed back to magnesium limitation (i.e. phosphate-replete conditions), the cells reverted back to a teichoic-acid-containing wall. In the case of the \textit{phoR} mutant (1A306), neither APase activity nor teichuronic acid synthesis was induced in response to phosphate limitation (Fig. 7b). The concentration of teichoic acid in the walls initially fell, but recovered, showing that in the absence of teichuronic acid synthesis, the cell is able to synthesize teichoic acid under phosphate limitation. Similar data were obtained for a \textit{phoP} mutant (data not shown).

Interestingly, the chemostat cultures indicate that steady-state levels of APase and anionic polymers are never reached (Fig. 7). APase activity is switched off, as reported previously by Hulett \textit{et al.} (1994a). However, we have consistently shown that, 40–50 h after change-over to phosphate limitation, both \textit{phoR} and \textit{phoP} mutants co-ordinately induce the synthesis of APase and teichuronic acid.

The data above suggested that the repression of teichoic acid synthesis during phosphate stress may require, at least in part, the synthesis of teichuronic acid. Further evidence comes from the analysis of the cell walls of a \textit{gtaB} mutant (GCH635). The inability of \textit{gta} mutants to synthesize UDP-glucose means that they synthesize non-glucosylated teichoic acid and are unable to synthesize teichuronic acid or the galactosamine-containing teichoic acid normally found in strain 168 (Archibald \textit{et al.}, 1993).

The anionic polymer composition of the cell walls of strain GCH635 was determined during steady-state growth in the chemostat under phosphate-replete (magnesium-limited) and phosphate-limited conditions (Table 4). Under phosphate-replete conditions, GCH635 synthesized approximately normal amounts of teichoic acid and no teichuronic acid was detected (Table 4). The walls of phosphate-limited GCH635 contained, as expected, no teichuronic acid but resembled the \textit{phoR}12 mutant in continuing to synthesize teichoic acid, in this case to about 60% of the level observed in phosphate-replete cells.

### DISCUSSION

\textit{phoR} mutants were used to study the response of \textit{B. subtilis} to phosphate stress, including the involvement of the Pho regulon in controlling the synthesis of anionic cell wall polymers. We have isolated and characterized a mutant that synthesizes a derivative of PhoR that is functionally active under phosphate-replete conditions. It shows increased synthesis of APases, APDase and teichuronic acid under phosphate-replete conditions. In contrast, a previously isolated and well-characterized \textit{phoR} mutant (1A306; Piggot & Taylor, 1977) with impaired PhoR activity failed to induce the synthesis of APases and teichuronic acid after transition to phosphate limitation.

In \textit{E. coli} PhoR is an HPKase required for the activation of the Pho regulon. In addition to phosphate-sensing activity, it is thought to exhibit both HPKase and PTase activities (Makino \textit{et al.}, 1989). The latter activity appears to be directed towards itself and toward its partner in the two-component signal-transduction pathway, namely PhoB in \textit{E. coli} or PhoP in \textit{B. subtilis}. It is

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**Table 4.** Cell wall anionic polymer content of a \textit{gtaB} mutant and its parent after growth to steady state in a chemostat

<table>
<thead>
<tr>
<th>Strain</th>
<th>Limiting substrate</th>
<th>Content [(\mu\text{mol (mg cell wall)}^{-1})] of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Teichoic acid</td>
</tr>
<tr>
<td>168</td>
<td>(\text{Mg}^{2+})</td>
<td>1.60</td>
</tr>
<tr>
<td>GCH635</td>
<td>(\text{Mg}^{2+})</td>
<td>1.69</td>
</tr>
<tr>
<td>168</td>
<td>P</td>
<td>0.06</td>
</tr>
<tr>
<td>GCH635</td>
<td>P</td>
<td>0.99</td>
</tr>
</tbody>
</table>
not known how the contradictory HPKase and PTase activities of PhoR are controlled; allosteric changes triggered by different phosphate concentrations could result in a switch between the two activities or to an increase in one activity over that of the other.

A merodiploid phoR strain showed the expected induction of the Pho regulon, as monitored by APase activity. Induction of phoR in strain GCH873 (P_phopRphoR, P_spacphoR) in phosphate-rich medium failed to induce APase activity, indicating that the induction of the Pho regulon is independent of the level of PhoR synthesis.

Clones containing the wild-type B. subtilis phoR were difficult to maintain in E. coli and we were never able to establish clones containing the intact phoR12 gene. The lesion in phoR12 was identified as a single base change leading to an Arg to Ser transition at position 532 (Seki et al., 1988). This residue is located between two regions that are conserved in the sensing components of many signal-transduction systems (regions 4 and 5; Albright et al., 1989; Lee et al., 1989), and within a region that is highly conserved in PhoR proteins (Fig. 5). The altered residue is the second Arg of a sequence Arg-Ser-Arg, that is, with the exception of the Synechococcus sp. protein (Aiba et al., 1993; GenBank U38917), highly conserved in PhoR proteins, including those of B. subtilis, E. coli, Klebsiella pneumoniae, Mycobacterium leprae and Shigella dysenteriae.

When strain GCH871 (P_phopRphoR12, P_spacphoR) was grown under phosphate-replete conditions, APase and, to a lesser extent, APDase were constitutively expressed. However, if IPTG was added to the culture medium, expression of the wild-type phoR suppressed the phoR12 lesion, indicating that the phosphatase activity of the PhoR leads to the dephosphorylation of PhoP. These data also suggest that: (i) the lesion in phoR12 affects its PTase activity; (ii) under phosphate-replete conditions both PTase and HPKase activities are present, but that the former is predominant; (iii) during phosphate stress, PhoR elaborates only HPKase activity. A mutant with a similar phenotype has been isolated for E. coli phoR (Scholten & Tommassen, 1993), although in this case the lesion was in domain C2, thought to be responsible for signal-sensing.

In strains of B. subtilis starved of phosphate, wall teichoic acid is replaced by teichuronic acid (Lang et al., 1982), a non-phosphate-containing anionic polymer. In batch cultures, GCH871 (P_phopRphoR12) showed increased teichuronic acid and decreased teichoic acid in cell walls, even under phosphate-replete conditions. This was confirmed in chemostat cultures of GCH872 (P_spacphoR12).

To determine whether PhoP/R was required for switching between these polymers, a previously characterized phoR mutant (1A306) was grown in chemostat culture under phosphate-limiting conditions. 1A306 was initially unable to induce teichuronic acid synthesis under phosphate limitation but instead continued to synthesize teichoic acid, albeit at a reduced level. Unexpectedly, we observed a reproducible induction of APase, an increase in teichuronic acid and a decrease in teichoic acid 40–50 h after changeover to phosphate limitation. A concomitant increase in phoA expression was confirmed by use of a phoA-lacZ fusion (data not shown). This ‘late’ response is reversed by the addition of phosphate, and cells subsequently isolated from the chemostat were found to respond normally with respect to the induction of APase during phosphate starvation. This observation implies an alternative mechanism for the induction of genes in the Pho regulon and is consistent with a previous report that some genes of the Pho regulon are controlled by a second, as yet unidentified, phosphate-sensing mechanism (Eymann et al., 1996).

The question arises as to whether the Pho regulon controls the synthesis of teichoic acid, teichuronic acid or both. phoR and gtaB mutants are unable to synthesize normal amounts of teichuronic acid under phosphate limitation, and both mutants compensate by continuing to synthesize teichoic acids, albeit at a reduced rate. These data suggest that teichuronic acid genes (tua) are under the control of the Pho regulon and that a component of teichuronic acid synthesis or an associated regulatory pathway may modulate the synthesis of teichoic acid. Such a mechanism, in which teichuronic acid rather than teichoic acid synthesis is subject to regulation by the Pho regulon, would provide an important fail-safe mechanism to ensure that the cells are provided with growth-sustaining amounts of anionic polymer.

The failure of strain 1A306 to replace teichoic acid with teichuronic acid was reflected in a twofold reduction in biomass in chemostat culture grown under phosphate limitation, compared with that of the wild-type. These data confirm the importance of the Pho regulon for survival during phosphate starvation.

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NOTE ADDED IN PROOF

Eder et al. (1996) recently reported the cloning and sequencing of phoD, encoding secreted APase and APDase activities.

REFERENCES


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